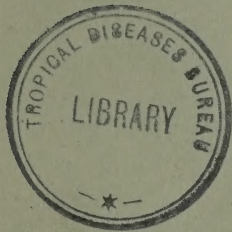
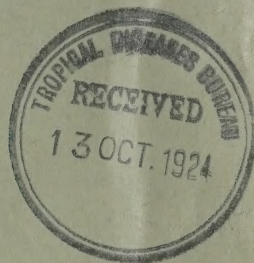


Summarized in V.D.B. Vol. 21. p. 726.

ON THE CULTIVATION AND BIOLOGICAL CHARACTERS OF LEISHMANIA TROPICA



BY
I. J. KLIGLER



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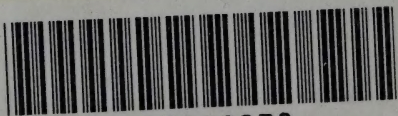
From the Laboratory, Rothchild Hospital, Jerusalem

REPRINTED FROM

THE AMERICAN JOURNAL OF TROPICAL MEDICINE

VOL. IV, No. 1, JANUARY, 1924

Made in United States of America



22200132378

ON THE CULTIVATION AND BIOLOGICAL CHARACTERS OF LEISHMANIA TROPICA

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From the Laboratory, Rothchild Hospital, Jerusalem

Received for publication August 11, 1923

INTRODUCTION

The prevalence of oriental sore is confined to certain endemic foci, which are, however, widely distributed throughout Asia. Forms of the disease exist in Bagdad, Delhi, Aleppo, Jericho, etc. Cases of Jericho boil are often seen in Jerusalem, and from time to time cases come in from other localities. Of the 7 cases studied during the past year, 5 were from Jericho, 1 from Aleppo, and 1 from Bagdad, hence there was opportunity to compare the characteristics of the lesions.

Character, number, and distribution of lesions

The lesions were always found on the exposed parts of the body. All of the patients had sores on some part of the face, 4 also had them on the hand; in only 1 patient were the lesions on the covered parts of the body. In the latter case there were 35 to 40 sores distributed all over the body, the first having appeared on the forehead. This instance was probably one of auto-infection.

Certain points of difference were noted between the "Jericho boil" and those of the patients from other localities. There were two or more boils in each of the 5 cases from Jericho, while the patients coming from Aleppo and Bagdad had only one lesion each. Cannan noted among the 26 cases of Jericho boil treated by him that a single boil was the exception, the multiple lesion being the rule. The Aleppo and Bagdad boils were flatter, red, and tubercular, while the Jericho boils were, as a rule, nodular

and smooth. Another point of distinction was the scarcity of *Leishmania* in the Jericho boils and their relative abundance in the lesions of the Bagdad and Aleppo patients. No differences were observed in the morphology of the parasites from the various lesions.

Endemic area and incubation period of Jericho boil

The endemic area of Jericho boil is apparently not limited to the city proper. One of the 5 cases studied had not been in Jericho but had worked for a month about 5 km. from the city, near the Dead Sea. We have records of other patients who have been in the vicinity of Jericho but not in the city itself. It is certain, however, that the disease occurs only within a relatively small radius about the city of Jericho.

The incubation period of the disease has not as yet been definitely determined. As a rule the patients do not come for treatment until long after the appearance of the lesions. In the cases studied it has been possible to establish a maximum and minimum incubation limit. All the patients had been in or near Jericho during the summer months, July to September, and began to come for treatment early in November. The infection was therefore contracted during the summer, and the lesions appeared early in the fall. The maximum period between the date of arrival in Jericho and the appearance of lesions was four months. In one instance the patient spent one month in Jericho, and the sores appeared one month after his return to Jerusalem. These facts indicate a maximum and minimum incubation time of two months and one month, respectively.

CULTIVATION EXPERIMENTS

Leishmania tropica was first noted by Wright (1) and first cultivated by C. Nicolle (2) on the N. N. N. medium, which has proved very satisfactory for the growth of the parasites, except that the cultures are relatively short-lived, hence require frequent transfer and do not lend themselves readily to a study of the biological characters of the parasites. In the course of the

present study a more suitable culture medium was sought, and it was found that excellent growth was obtained in a medium similar to that employed by Noguchi for the cultivation of *Leptospira icteroides* (3) except that rabbit whole blood is substituted for rabbit serum,¹ and that the agar used contains a small amount of dextrose.

The preparation of the medium is very simple. One per cent dextrose agar is melted, and 10 parts are mixed with 90 parts of saline. The diluted dextrose agar is now distributed into tubes (5 or 10 cc. per tube) and sterilized. This constitutes the basic medium and may be kept in stock indefinitely. Immediately before or just after inoculation fresh sterile rabbit blood is added to each tube in a proportion of 1 to 10.

With the medium just described successful cultures of *Leishmania* were obtained from lesions of patients from three different localities. Material from all boils which had not yet become infected with staphylococci yielded positive results, but whenever the lesion was already contaminated with this organism all efforts to sterilize it before taking material for culture proved unsuccessful. The procedure for taking the material for cultivation purposes was as follows: The surface of the boil and the surrounding skin were painted with tincture of iodine, then washed thoroughly with alcohol and ether. A capillary pipette was then inserted sidewise near the base of the boil and twisted about to break up the tissue. Active bleeding of the wound followed, and small amounts of material were taken up by the capillary and inoculated into the culture tubes. Sometimes small bits of tissue were excised, macerated in citrated saline, and inoculated into culture medium. The culture tubes were covered with sterile paraffin oil and incubated at a temperature of about 25°C.

Cultural properties of Leishmania tropica

The success of the cultures can be followed macroscopically. At the time of inoculation the tubes have the bright red color

¹Since 1920 Noguchi has been adding a small amount of rabbit hemoglobin regularly to the medium used for the cultivation of organisms of the *Leptospira* group.

of oxygenated blood. If the culture remains free from contaminating organisms, the color of the medium darkens at the bottom, but the zone near the top remains bright red. Contaminating organisms bring about changes in the color of the blood, hemolysis, etc., which can be easily detected after three or four days. Such tubes may be discarded, since *Leishmania tropica* rarely grows in the presence of contaminating bacteria. Tubes which remain free from bacteria gradually undergo hemolysis, and a positive culture can almost invariably be recognized by the bluish black appearance of the medium and the watery bluish tint of the paraffin oil when looked at from above.

In positive cultures flagellated forms may be found, by careful darkfield examination, on the third or fourth day. If the inoculated material contained only a few organisms, a week or more may elapse after inoculation before the parasites can be detected by darkfield examination.

In fresh cultures the various flagellated forms described by other observers are noted; as the culture ages more and more ovoid forms are seen, and in old cultures the typical non-flagellated ovoid parasites, such as are found in the lesion, are encountered.

The organisms are particularly abundant in bits of fibrin, large colonies being entangled in the fibrin network, which seems to offer a favorable substratum.

Leishmania tropica is an obligatory aerobe. Experiments made with different oxygen tensions showed that active continued growth can be obtained only at normal oxygen tension. Under anaerobic conditions no growth is obtained. Paraffin oil does not modify the aerobic condition of the medium.

Leishmania tropica is very sensitive to changes in reaction. At pH 7.8 and 6.6, respectively, there was no growth at all. Growth was obtained in the range from pH 7.0 to 7.6 but was best at pH 7.2. The tests were made in media of which the reaction was adjusted by means of HCl and acetic acid, unbuffered, and buffered with phosphate, always with the same results.

Positive cultures were obtained from three cases. All the negative cultures were contaminated with staphylococci. Of

the positive cultures, the one from the Aleppo boil was carried to the third generation in a period of four months and lost by accident in the incubator, that from the Jericho boil was carried two generations in three months and was lost through mould contamination; the third, from the Bagdad boil, has now been carried a year through seven generations.

Routine transplants are usually made about every six weeks, but examinations and subcultures have been made from time to time for the purpose of studying the viability and morphological changes of the organisms. The initial cultures were found to be viable after two months. Second and third generation cultures were active two and one-half and three and one-half months after transplantation. The fifth and sixth generations also contained actively motile forms after three and three and one-half months. If the culture escapes the contamination by moulds to which cultures are exposed in hot climates, and the incubator is kept between 22° and 25°C., the culture will easily survive three months and probably longer. The paraffin oil prevents evaporation of the medium and rapid changes in reaction. Buffering with peptone solution helps to preserve the culture (4), but no appreciable differences were noted between buffered and non-buffered cultures.²

Germicidal action of certain substances on Leishmania tropica

The substances tested for their germicidal effect on the organism were salvarsan, antimony, and brilliant green. The experiments, which will be repeated and extended, are recorded in tables 1, 2, and 3.

The procedure was as follows: Various concentrations of the substance to be tested were added to the usual culture medium in a series of tubes, which were then inoculated with a uniform amount (0.1 cc.) of culture and incubated. Examination was made at regular intervals.

² When serum is present in the culture medium, it undergoes rapid alkalization, and the duffer is therefore useful. In these cultures no serum was added, and the acidity of the laking blood plays the part of buffer.

In table 3 is recorded an experiment to test the germicidal power of tartar emetic. Graduated amounts of a 1:1,000 solution of tartar emetic were added to tubes containing 5 cc. of neutral ascitic fluid. One-tenth cubic centimeters of culture was then added to each tube, and the tubes examined at regular intervals under the darkfield microscope.

TABLE 1

Comparative effect of tartar emetic and brilliant green; results after two weeks incubation

TARTAR EMETIC			BRILLIANT GREEN			CONTROL
1:10,000	1:20,000	1:40,000	1:100,000	1:200,000	1:400,000	
—	—	+	—	—	+	++

TABLE 2

Results after two weeks incubation

	1:10,000	1:20,000	1:25,000	1:33,000	1:50,000	CONTROLS
Tartar emetic	—	+	++	++	++	++
Salvarsan	—	—	+	++	++	++
Neosalvarsan	—	—	—	+	++	++

+ = relatively abundant growth.

TABLE 3

TARTAR EMETIC	AFTER THREE HOURS	AFTER SIX HOURS	AFTER TWENTY-FOUR HOURS
1: 2,500	Active	Partially immobilized	—
1: 6,600	Active	Partially immobilized	—
1:10,000	Active	Active	—
1:20,000	Active	Active	Active
Control	Active	Active	Active

ADDENDUM

Since the foregoing report was written, it has been found that subcultures of *Leishmania tropica* in the regular leptospira medium of Noguchi (consisting of 8 parts 0.9 per cent salt solution, 1 part rabbit serum, 1 part 2 per cent nutrient agar, and 0.2 part rabbit hemoglobin, made by adding 3 cc. distilled water to

1 cc. of rabbit blood) remain alive for at least six months without transfer. For subcultures, therefore, the leptospira medium is most suitable, while for primary cultures the modification described has given the best results.

SUMMARY

The successful cultivation is reported of the *Leishmania* of oriental sore in a medium similar to that used by Noguchi for the cultivation of *Leptospira icteroides*. Whole blood is used in place of serum, and the agar employed contains a small amount of dextrose.

The medium is prepared by adding one part of dextrose agar to nine parts of saline. The semisolid agar is then tubed and autoclaved. Before use, 0.3 to 0.5 cc. of fresh rabbit blood is added to each tube.

Cultures were obtained from 3 cases of oriental sore from three different sources and were kept alive for periods ranging from two and one-half to three and one-half months. One culture has now been carried through eight generations during a period of thirteen months.

SUMARIO

El objeto del presente artículo es el informar acerca de un nuevo procedimiento que permite cultivar los *leishmanias* que causan la úlcera oriental. El método de cultivo es muy parecido al que Noguchi usa para el *Leptospira icteroides* con la excepción de que en este caso se usa sangre completa en vez de suero.

El medio se prepara añadiendo a 1 parte de agar glucosada, 9 partes de solución salina fisiológica. Este agar semi-sólido se pone entonces en tubos y se esteriliza al autoclave. Antes de usarse se añade a cada tubo de 0.3 a 0.5 cc. de sangre fresca de conejo.

Con este medio se obtuvo el desarrollo artificial del organismo causante de la úlcera oriental de tres casos de la enfermedad, todos de procedencia distinta. Se mantuvieron vivos durante períodos que variaban de dos meses y medio hasta tres meses y medio. De uno de ellos se han hecho ocho transplantes, con éxito, durante un período de 13 meses.

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THE AMERICAN JOURNAL OF TROPICAL MEDICINE

VOLUME IV, NUMBER 1, JANUARY, 1924

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